BREATH ANALYSIS USING PHOTOIONIZATION MASS SPECTROMETRY

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ABSTRACT

REMPI (Resonantly Enhanced Multi Photon Ionization) is an ultra-sensitive analytical technique based on the unique combination of laser spectroscopy and mass spectrometry that can rapidly identify and quantify vapor-phase constituents present at part per trillion levels. We have used this method to examine human breath samples for biomarkers of infection. Based on the observed results, we estimate our limit of detection for the photoionization system is a factor of 40-100 times lower than we can achieve using the newest GC/MS instrument. Of more importance perhaps, compounds such as benzene, toluene or even dichlorobenzene can readily be detected using this method.

INTRODUCTION

The threat posed by the use of biological weapons against military or civilian targets and their potential for mass destruction form a major national concern. Developing an appropriate response involves first detecting the use of a chemical or biological warfare (CW/BW) agent. This can be accomplished either by direct detection of the agent or by diagnosing the health of the affected persons. We have been investigating the latter approach through the use of volatile biomarkers in exhaled breath. These compounds can be produced by the infectious biological agent or through the host response. From the small amount of previous work in this general area, we anticipate that different suites of such compounds will be associated with specific diseases. One of the chief technical challenges to the use of breath analysis as a health diagnostic in general, and a monitor for BW exposure in particular, is a rapid, sensitive, and chemically selective detector.

Given the nature of most BW-induced diseases, it is crucial to detect them very early in the infection stage. When typical symptoms become apparent, it is often too late to apply traditional countermeasures, i.e., the administration of antibiotics and antidotes. However, when countermeasures are applied early, they can be quite successful. Thus, we have focused on the detection and identification of biological activity indicating exposure and the early onset of disease.

We describe here the use of SRI's ultratrace detection system, already developed and tested for other applications. This system consists of a tunable, narrow-band, laser and a time-of-flight mass spectrometer to rapidly and selectively measure compounds in the breath with very high sensitivity. Specific compounds found in exhaled breath are known to be associated with specific diseases and can, in principle, be used as a noninvasive medical diagnostic. Little progress has been made in this field, and conclusions have been hampered by ambiguous results, perhaps due to the lack of sensitivity of the conventional analytical methods used in these studies.[1] Our ultratrace method can already rapidly detect

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many organic compounds, especially those with an aromatic ring, at the part-per-trillion (ppt) level in 10's of seconds, which is beyond what is currently available.

Our goal is the early identification of specific diseases by analyzing the exhaled breath of individuals who may have been exposed to infectious agents. The specific analysis of compounds in the breath for disease diagnosis is in its infancy, yet useful conclusions have been made with detection sensitivity orders of magnitude poorer than ours. We anticipate that the increased sensitivity using our method will enable detection of disease onset at the most incipient stage, perhaps hours or even days before conventional symptoms are present. This early detection will allow countermeasures to be applied in the most effective time period.

Moreover, this approach can be applied to many diseases, not just those caused by BW agents. Biological activity induced by sources such as CW agents or toxic chemicals could also be detected. Thus, the technique could find use in civilian hospitals and clinics as a rapid, noninvasive method for disease diagnosis.

Trace compounds in exhaled human breath reflect both physiological state and exposure to the surrounding environment. Therefore, the trace organic constituents of expired breath can provide a useful index of exposure or disease. The breath analysis of volatile organic compounds (VOCs) opens a noninvasive window to normal metabolic pathways and also illustrates how these pathways are altered by exposure or disease.

Human breath contains several thousand VOCs in low concentrations.[2,3] Many of these are emitted under normal physiological circumstances, and others only when disease is present. The latter can be new compounds produced by the invader and/or the host, or elevated levels of those normally present. Toxic VOCs can also be found in exhaled breath after inhaling polluted air. There have been many studies on this last topic, in large part because the compounds are present at relatively high concentrations. In contrast, few studies has been done on infectious disease-produced organic compounds, which may be present at very low concentrations.

In seeking a disease-specific biochemical marker, one is confronted with the problem of identifying a specific marker among several hundred or more similar components, all of which are present in microgram per liter to sub-microgram per liter concentrations (ppb or below). Despite this analytical challenge, several correlations between diseases and biomarkers have been reported, e.g., correlations between lung cancer and aniline and o-toluidine, as described below.[2] Because many of the components present in human breath may not be relevant for medical diagnostic purposes, a successful diagnostic technique must be capable of selectively identifying unique species in this complex matrix and rapidly monitoring changes at very low concentrations.

Breath analysis has a long history of application to detection of disease. Before the development of modern analytical instrumentation, physicians used qualitative descriptions of breath odor to diagnose disease. Terms for breath odors have found their way into common medical vocabulary, such as uremic breath, diabetic breath, and fetor hepaticus. Modern instrumentation has allowed establishment of relationships between disease and specific molecules in breath.[3] It is not essential that the disease specific markers be produced in the respiratory system to provide a marker observable through breath analysis. Furthermore, many effective BW agents are introduced into the body through respiration, so breath analysis has more immediate connection with the initial disease site, enhancing early detection.

Examples of markers produced by the host response to an infectious or toxic agent include those listed for uremia and cirrhosis, which may be caused by Escherichia coli[4] or hepatitis C infection,[5] respectively. Thus, host-induced markers may provide information specific to the infectious agent or to the organs or regions of the body affected by an infectious or toxic agent.

Breath analysis can be used for applications other than detection of disease biomarkers. In fact, the most widely known type of breath analysis is the breathalyzer test used to measure breath alcohol

(ethanol) levels. Breath analysis has also been used to detect a variety of drugs, including delta-9-tetrahydrocannabinol (THC), anesthetics, and disulfiram (Antabuse). Most of these tests monitor the primary molecular compound associated with the drug. However, interactions of the human body with the drug have also been demonstrated, e.g., breath detection of carbon disulfide produced from disulfiram[6] and acetaldehyde from alcohol.[7] As for disease detection, breath detection can monitor either a primary molecular marker or secondary markers produced in vivo.

Breath analysis is also widely used to monitor the uptake of toxic substances or the response of the body to toxic substances or other insults.[3,8] It can be used to detect the primary compound, as for detection of benzene[9] or styrene,[10] or to detect physiological changes, as has been demonstrated for alterations in breath composition after inhibition of cytochrome P450 by using trans-1,2-dichloroethylene (DCE).[11]

EXPERIMENTAL METHOD

Our system is an ultrasensitive analytical technique that can identify and quantify vapor-phase constituents present at part per trillion levels in the human breath rapidly and without the need for sample collection, preconcentration, or pretreatment. The real-time nature of the instrument allows these measurements to be made in minutes or even seconds and therefore can diagnose a disease in a fraction of the time it takes today (hours to days). Measurements may also be made as a function of time (for example, on an hourly basis or over many days) to observe fluctuations due to disease development as a function of drug treatment, change of drugs, drug concentration, and other conditions.

The instrument package of the system is known in the scientific literature as Jet-REMPI. It has been used for over a quarter century for fundamental studies of molecular spectroscopy, molecular structure, and chemical reactions. Resonantly enhanced multiphoton ionization (REMPI)[12,13] is a highly sensitive, highly molecule-specific analysis technique that is starting to be applied to problems in analytical chemistry, molecular spectroscopy, and combustion diagnostics.[14] Even the exhaled breath of smokers was investigated successfully in recent years.[15] SRI has been using this laser-mass spectrometric technique to study spectroscopy and trace-level detection for more than a decade.[16]

The REMPI approach involves the absorption of a first photon that excites a target molecule from its ground state, S_0, to the first excited state, S_1, and absorption of a second photon that ionizes the molecule. This method of ionization produces solely or predominantly the parent molecular ion, which is then detected using a time-of-flight mass spectrometer. The selectivity of ionization arises from the resonance of the first step. If the laser is detuned from this resonance, the probability of ionization drops by many orders of magnitude. Thus, by proper choice of the laser wavelength for REMPI, only specific molecules will be ionized—those that have a resonant level at the energy of the laser photons.

The ionization efficiency for Jet-REMPI can be extremely high, and ionization efficiencies from 1% to 10% have been reported.[17] Many rovibrational levels in the excited state can be used for REMPI. Choice of the level is influenced by the transition strength, the ground state population, the excited state lifetime, the energy gap to the ionization potential, and the potential for fragmentation.

The sensitivity and specificity for ultratrace detection can be dramatically improved when it is performed using cooling with a supersonic jet (hence the name jet in the scientific literature term "Jet-REMPI"). In a supersonic jet, the adiabatic expansion leads to dramatic cooling, providing temperatures down to approximately 18 K.[18] This cooling greatly simplifies the spectra, producing narrower and stronger electronic transitions. The lower gas temperatures lead to population of fewer rovibrational levels, which in turn produce larger peak absorptions. This effect improves the sensitivity because there is more population in the absorbing levels, providing larger signals. Selectivity is improved because there are fewer absorption lines of other molecules that might interfere with the absorption lines of the target molecules.

Our Jet-REMPI apparatus is shown schematically in Figure 1. Samples are introduced through a pulsed valve into vacuum. The molecules, cooled by the jet expansion, are ionized with light from a tunable optical parametric oscillator (OPO). Tuning the OPO wavelength selects different molecules for ionization. Ionized molecules are detected by the time-of-flight (TOF) mass spectrometer. This simultaneous, two-dimensional, identification through the combination of laser absorption spectra and mass determination produces our unprecedented selectivity at low concentrations.

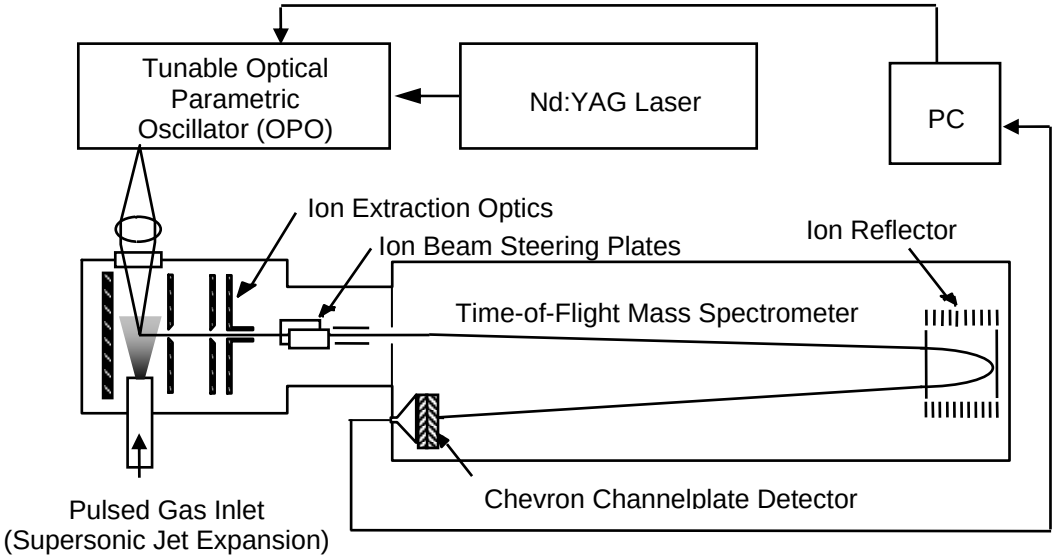

Figure 1. Jet-REMPI apparatus.

RESULTS AND DISCUSSION

To demonstrate the ability of our ultratrace detection method to detect potential biomarkers, we have investigated aniline and o-toluidine. Both molecules are indicated as markers for lung cancer,[2] and the combined detection of both should provide even more definitive confirmation of the presence of the disease.

This work, performed recently in our laboratory, is the first study of these compounds in mixtures. Aniline was detected by this approach earlier for spectroscopic purposes, but o-toluidine had never been studied. Aniline and o-toluidine were measured in a synthetic mixture in order to prove the selectivity of the ultratrace method by detecting the target molecules in the presence of similar compounds at excess concentrations. The concentrations for aniline and o-toluidine in these measurements were each about 30 ppb. The mixture includes as the main carrier laboratory air (not usually clean itself!), to which we added 0.1% acetone and 1 part-per-million (ppm) concentrations of benzene, toluene, p-xylene, chlorobenzene, and p-cresol. We anticipate that these relative concentrations are indicative of the kinds of mixtures of many compounds found in human breath.

Although the mass spectrometer records all masses simultaneously, we can display only the parent masses corresponding to aniline and o-toluidine. The OPO is then scanned, producing spectra that are definitive signatures of the compounds. The spectra for the two compounds are shown in Figures 2 and 3.

As noted, these measurements were made at approximately 30 ppb of each target compound in a mixture. Because these were our first measurements of these two compounds, the system was not fully optimized for their detection. From our more extensive work on other similar compounds, we anticipate a

limit of detection of a few parts per trillion of each of these molecules with an averaging time of a minute or less.

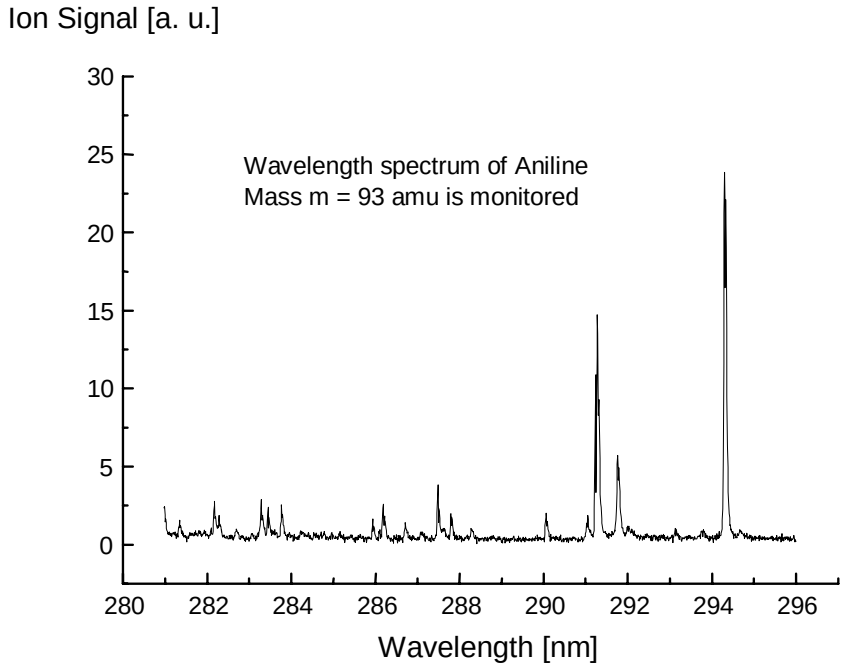

Figure 2. Wavelength spectrum of aniline.

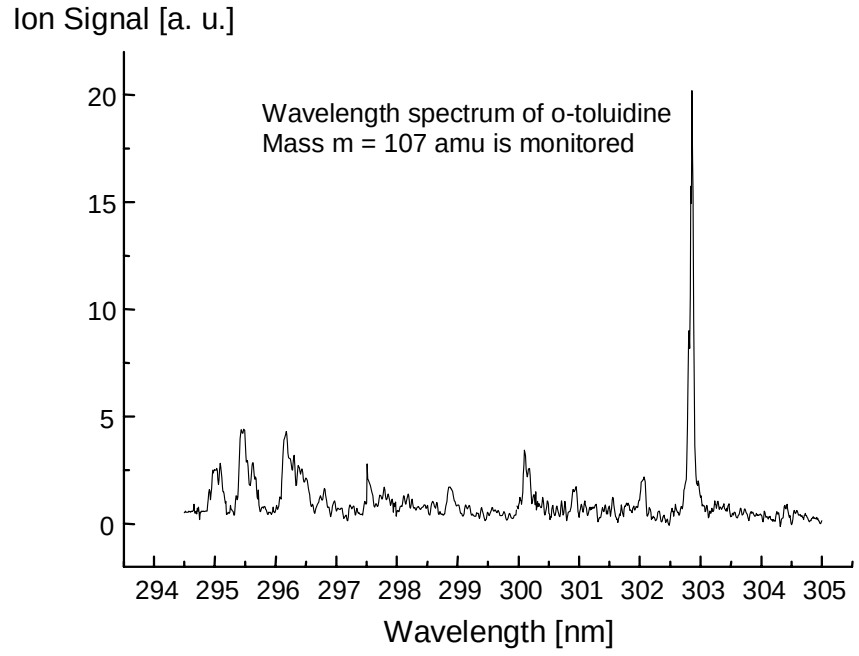

Figure 3. Wavelength spectrum of o-toluidine.

The first step in doing the photoionization work with expired human breath was to establish a uniform sampling protocol that would collect and release representative vapors. We determined that the Solid Phase MicroExtraction (SPME) fibers used to collect breath from infected piglets are the most reliable and straightforward to use.[19] Following an IRB approved human subjects plan, we acquired a breath sample from an SRI volunteer. Two 30-second breaths were collected on a SPME fiber under conditions that minimized collection of room background vapors. Naturally, volatiles in the room air were inspired and subsequently expired making an independent examination of the room air necessary. Because the composition of both the expired breath and the room air were unknown and since REMPI is not a general survey tool, we employed standard gas chromatography/mass spectrometry (GC/MS) analysis to ascertain composition. The SPME fiber was desorbed in the inlet of the Agilent 6890/5973 GC/MS, with the total ion chromatogram shown in Figure 4. Note that a number of significant peaks are known to be associated with the fibers themselves and are not identified. Furthermore, all peak identifications were made by searching the NIST MS library and selecting the most realistic compound whose mass spectra fit the measured signal.

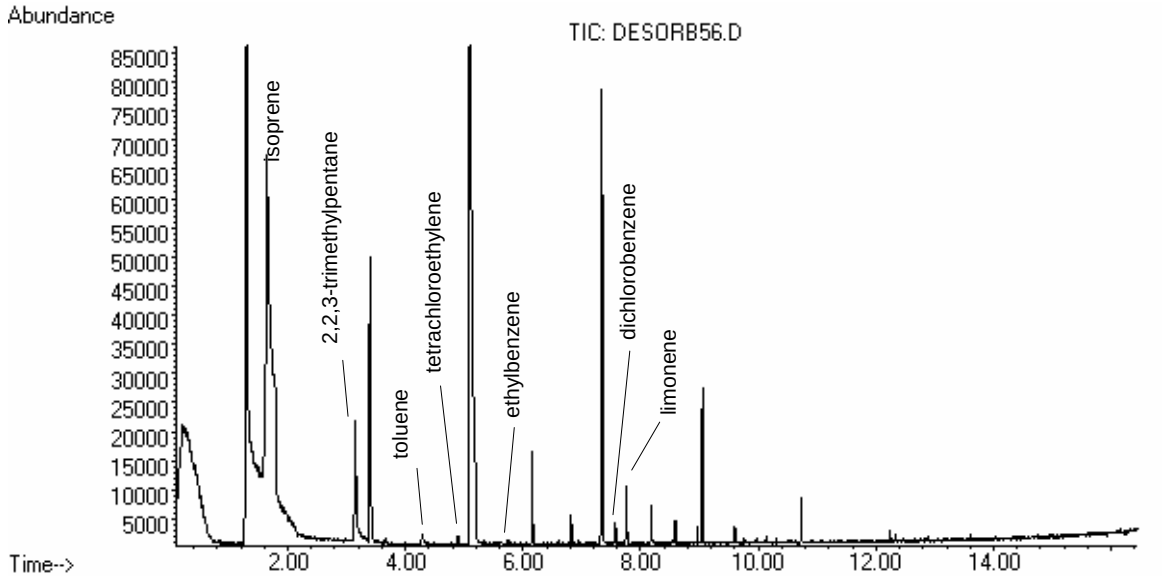

Figure 4. Total ion chromatogram from human breath collected from an SRI volunteer.

The major volatiles identified from the TIC in Figure 4 are isoprene, 2,2,3 trimethylpentane, toluene, tetrachloroethylene, ethylbenzene, dichlorobenzene, and limonene (note that several of these peaks appear very minor but that is a result of the scaling – they are all well above the noise background). Of these, only toluene and limonene are found in a room air background collected under similar sampling and analysis conditions. Of the remaining compounds, the most surprising was dichlorobenzene. Because dichlorobenzene is not a common air toxic or industrial pollutant, its presence was difficult to rationalize until the subject mentioned that they had a paper bag full of mothballs in their bedroom closet. An independent vapor analysis by GC/MS and REMPI verified that the mothballs were emitting significant levels of dichlorobenzene. The subject has since removed the mothballs from their closet and we have subsequently acquired several additional breath samples from the volunteer over a 3-month period. Each successive sample has shown that, although the dichlorobenzene remains in their expired breath, its level is decreasing.

The duplicate breath sample from the same volunteer was examined using the photoionization instrument. Because we wanted to see as many volatile aromatics as possible from this breath sample, we configured our instrument to perform multiphoton ionization using a fixed wavelength of 266 nm. This

well-established method gives very efficient ionization of aromatic compounds and does not required scanning the laser. This is particularly important when using the SPME fiber as the sample is present in the inlet for only 15 seconds during the thermal desorption which is insufficient to perform a wavelength scan required to do a resonant (REMPI) ionization survey. Since we were interested in seeing the dichlorobenzene signal, this approach was reasonable. When using a fixed wavelength ionization scheme, there is no benefit to using a pulsed gas inlet to internally cool the sample molecules. Hence, we used a conventional GC injector with a direct capillary inlet to desorb and transfer the sample from the fiber to the photoionization region.

The result of our very first photoionization analysis of human breath is shown in Figure 5. Here, we show the summed mass spectrum acquired over a period of 10 seconds (100 laser shots), and a corresponding laboratory air background spectrum. A number of mass peaks appear in both the breath sample and the background (benzene, toluene, and xylene), while some aromatics appear only in the background (phenol, cresol). Likewise, a set of mass peaks corresponding to dichlorobenzene is readily observed only in the breath sample as indicated in Figure 5.

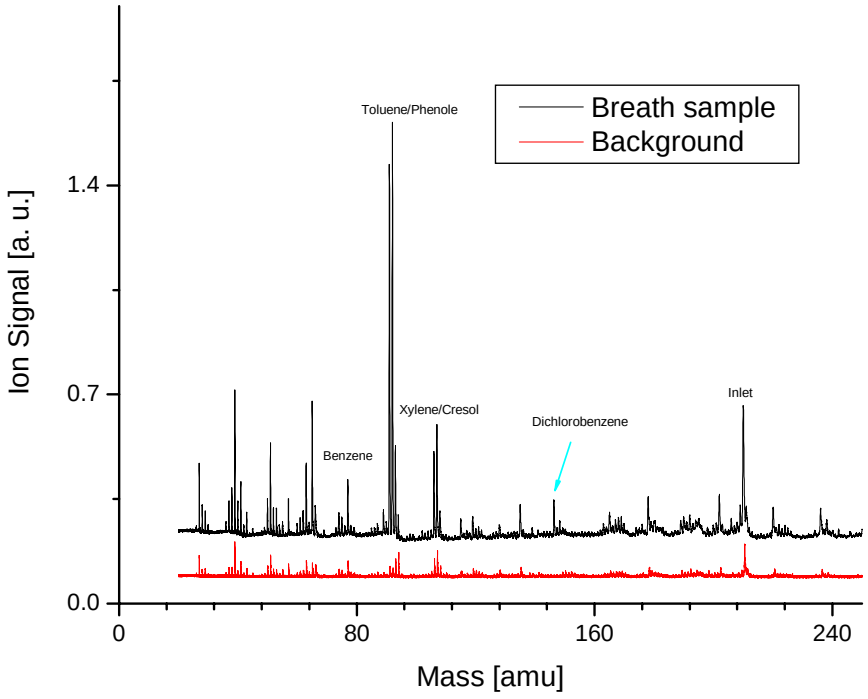

Figure 5. Photoionization (266 nm) mass spectra of breath and room air background.

To demonstrate the rapid nature of the photoionization system, we removed the septum from the injection port and allowed the subject to exhale in the vicinity of the open capillary. We recorded the dichlorobenzene parent ion peak as a function of time as shown in Figure 6. The data clearly show the spikes in the signal corresponding to each exhaled breath. The variation in intensity is likely due to the open capillary sampling resulting in a variable collection efficiency.

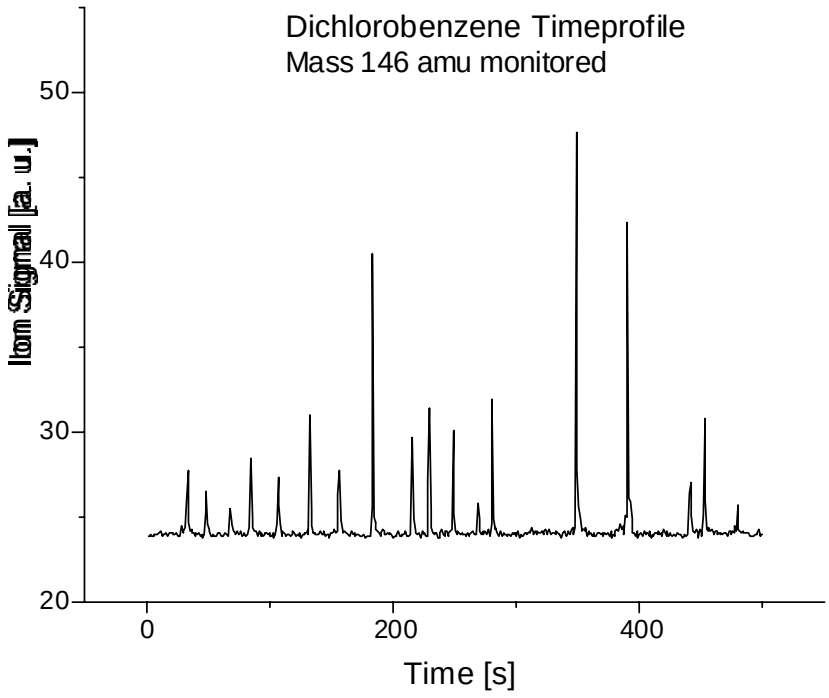

Figure 6. Dichlorobenzene REMPI signal as a function of time with direct sampling.

CONCLUSIONS

Based on the observed S:N ratio and instrumental gain settings of the photoionization system, we estimate our limit of detection for dichlorobenzene is a factor of 40-100 times lower than we can achieve using the newest Agilent GC/MS instrument. Of more importance perhaps, a compound such as dichlorobenzene could readily be detected using our method in real time directly in expired breath, that is, without collection on a SPME fiber. The same holds true for all of the aromatic compounds seen in Figure 5.

This unexpected, and very interesting, preliminary experiment served to: (1) establish sampling via SPME fiber as a useful technique, (2) confirm that fixed wavelength photoionization is a viable means of detecting trace aromatics in expired human breath, (3) verify that photoionization is more sensitive that GC/MS for these compounds by a factor of 1-2 orders of magnitude, and (4) at least for aromatic vapors, direct, real-time breath analysis is possible using our instrument.

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